

Updated Distribution of Anopheline Mosquitoes (Diptera: Culicidae) in Hokkaido, Japan, and the First Evidence of Anopheles (Anopheles) Belenrae in Japan

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Abstract

Background: In Hokkaido, northern island of Japan, at least seven cases of falciparum malaria were reported by 1951. A survey conducted at that time was unsuccessful in implicating any mosquito species as the possible vector. Although active anopheline mosquito surveillance continued until the middle of the 1980s, there is very limited information on their current status and distribution in Japan. Therefore, this study is an update on the current status and distribution of anopheline mosquitoes in Hokkaido based on a 15-year entomological surveillance between 2001 and 2015.

Methods: A survey of mosquitoes was conducted at 22 sites in Hokkaido, Japan, from 2001 to 2015. Adult mosquitoes were collected from cowsheds, lakesides, shrubs, and habitats ranging from open grassland to coniferous forest using a CDC miniature light trap enhanced with dry ice, aspirators, and sweeping nets. Larvae were collected from lakes, ponds, swamps, stagnant and flowing rivers, and paddy fields. All specimens were morphologically identified and subjected to PCR-based sequence analysis of the ITS2 region of rDNA. Phylogenetic trees were reconstructed using the neighbor-joining method.

Results: A total of 46 anopheline specimens were used for the phylogenetic analysis. During the survey, a new member of the *Anopheles hyrcanus* group, *An. belenrae* Rueda (2005), was discovered in eastern Hokkaido in 2004. *Anopheles belenrae* has since then been consistently found and confirmed to inhabit only this area of Japan. Four members of the *An. hyrcanus* group, *An. belenrae*, *An. engarensis*, *An. lesteri*, and *An. sineroides*, have been found in Hokkaido. The results also suggest that *An. sinensis*, formerly a dominant species throughout Japan, has become a rarely found species, at least currently in Hokkaido.

Conclusion: The updated distribution of anopheline mosquitoes in Hokkaido, Japan, showed considerable differences from that observed in previous surveys conducted from 1969 to 1984. In particular, areas where *An. sinensis* was previously distributed may have been greatly reduced in Hokkaido. The phylogenetic analysis revealed a novel *An. hyrcanus* group member identified as *An. belenrae*, described in South Korea in 2005. It is interesting that *An. belenrae* was confirmed to inhabit only eastern Hokkaido, Japan.

Background

After World War II, both postwar malaria and endemic malaria were prevalent in many areas of Japan.

Reported cases reached 28,000 annually in 1945 and 1946, with over 7,000 cases of vivax malaria up to the end of the 1950s. Surprisingly, at least seven cases of falciparum malaria were reported between 1947 and 1951 in Rubeshibe, Hokkaido (43.78 N, 143.61 E), located in the north of Japan. Although a survey was conducted to determine the vector mosquitoes involved at that time, no suspected species were found [1]. Endemic malaria was considered eliminated by 1960. The number of malaria cases has decreased drastically since then, with less than 80 imported cases annually in the past 10 years: 20 imported cases in 2020 [2].

Anopheles species contain the most important malaria vector species. Among those recorded in Japan, *An. sinensis* Wiedemann, 1828 is the most widespread and common anopheline species. This species is considered the major vector of vivax malaria in Korea and China. Previous surveys conducted in Japan revealed that *An. sinensis* was the dominant anopheline species in Japan, including Hokkaido; *An. lesteri* Baisas & Hu, 1936 was commonly found in Hokkaido with only a few *An. sineroides* Yamada, 1924 [3–6]. These surveys also found a new member of this group, *An. engarensis* Kanda and Oguma, 1978 [3–5]. Thus, several malaria vector species (e.g., *An. sinensis*, *An. engarensis*, and *An. lesteri*) continue to inhabit Japan. Despite the need for a nationwide survey to systematically assess these species, very little information is available, mostly gathered in the 1980s. Recently, several DNA barcoding projects have been conducted on mosquitoes in Japan, and a small number of genomic information on anopheline mosquitoes were included [7–9]. However, these studies were not specific to malaria vector mosquitoes.

At the onset of this survey, the distribution of five species of the *An. hyrcanus* group, *An. sinensis*, *An. sineroides*, *An. lesteri*, *An. engarensis*, and *An. yatsushiroensis* Miyazaki, 1951, had been confirmed in Japan. Moreover, of these five species, only *An. yatsushiroensis* has never been reported in Hokkaido [10–13], the region of interest in this study. Nonetheless, the highly similar morphological features of the members of this group, particularly *An. engarensis* and *An. sinensis*, makes it difficult to distinguish between species morphologically. Therefore, the frequency of clasper movements in males, hybridization studies, and chromosomal studies were used in distinguishing *An. engarensis* from the Japanese population of *An. sinensis* [3–5]. Recently, they have effectively been identified using polymerase chain reaction (PCR) and sequence analysis. Among the molecular markers used for mosquito taxonomy, the cytochrome oxidase subunit 1 (COI) sequences of the DNA barcoding region [14–16], and the internal transcribed spacer 2 (ITS2) region of rDNA are the most efficient. ITS2 in particular, is very efficient in distinguishing between closely related species, e.g., *An. maculipennis* complex, *An. quadrimaculatus* complex, *An. culicifacies* complex, and *An. gambiae* complex [17–20]. ITS2 has also been used to address taxonomic issues in the *An. hyrcanus* group [21–26].

For about 20 years since the last survey in 1984 [6], very few surveys of malaria vector mosquitoes had been conducted in Japan. We therefore initiated nationwide surveys from 2001 to determine the current status and distribution of anopheline mosquitoes in Japan. During our survey, we recorded a new member of the *An. hyrcanus* group in eastern Hokkaido in 2004. They were genetically confirmed to be *Anopheles (Anopheles) belenrae* Rueda (2005), described in South Korea in 2005 [24]. In the present study, species identification and determination of genetic distances between specimens was carried out by analyzing ITS2 region. Special attention was given to determining the distribution of *An. belenrae* in Japan. Finally, we updated the information from previous surveys [3–6] on the current distribution of the anopheline mosquitoes in Hokkaido.

Materials And Methods

Mosquito sampling

Mosquitoes were collected at 22 sites in Hokkaido from 2001 to 2015 (Tables 1 and 2). In this study, eight specimens collected in domestic areas outside Hokkaido were used as a reference specimen in phylogenetic analysis. Seven of the eight specimens were from Japan and the last from Vietnam. The areas in Japan and year surveyed were Kanagawa Prefecture in 2001, Akita Prefecture in 2005, Aomori and Toyama Prefectures in 2007, and Gifu, Fukui and Tokushima Prefectures in 2009. The specimen collected in Vietnam, Gia Lai Province in 2007, served as an outside Japan *An. sinensis* reference strain. Details of the collection sites are provided in Table 2.

Adult mosquitoes were collected from cowsheds, lakesides, shrubs, and habitats ranging from open grassland to coniferous forest throughout the day using a CDC miniature light trap enhanced with dry ice [27], aspirators, and sweeping nets for approximately 3 h after sunset. Collected adult mosquitoes were frozen and transported in an icebox to the National Institute of Infectious Diseases (NIID), Tokyo, Japan. Larval mosquitoes were collected from paddy fields, swamps, stagnant and flowing rivers, lakes, and ponds using dippers. Larvae were transported alive to NIID and reared to adults under laboratory conditions of 25°C, 60–70% relative humidity, and a photoperiod of 16:8 (L:D) h. Morphological identification was performed on all adult individuals using taxonomic keys [11, 28]. All classified mosquito specimens were transferred individually into 1.8 mL microtubes (Eppendorf, Hamburg, Germany), and stored at -80°C until subsequent analyses by ITS2 sequencing.

DNA extraction, ITS2 amplification and sequencing

Total genomic DNA was extracted from individual samples using a REExtract-N-Amp Tissue PCR Kit (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's protocol. Extracted mosquito DNA was subjected to PCR-based sequence analysis and phylogenetic analysis using primers of the ribosomal DNA ITS2 region (forward, 5'-TGT GAA CTG CAG GAC ACA-3'; reverse, 5'-TAT GCT TAA ATT CAG GGG GT-3') [29]. Amplification conditions were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a 4 min final extension at 72°C using a Veriti™ 96-well Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA).

All visible PCR-amplified DNA fragments were purified using the Qiaquick PCR Purification Kit (QIAGEN, Venlo, Netherlands) or extracted using MonoFas (GL Sciences Inc., Tokyo, Japan) from a 2% low melting point agarose gel (SeaPlaque GTG agarose, Cambrex Corp., East Rutherford, NJ) after preparative gel electrophoresis and visualization with ethidium bromide. Each purified double-stranded PCR product was directly cycle-sequenced from both ends using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit v3.1 (Thermo Fisher Scientific Co.) and the PCR-primers [29]. The thermal profile used was 25 cycles of 96°C for 10 s, 55°C for 5 s, and 60°C for 4 min using a Thermal Cycler, and ABI PRISM 3730 Genetic Analyzer (Thermo Fisher Scientific Co.).

Sequence analyses was performed using the GENETYX software ver. 14 (Genetyx Corp., Tokyo, Japan). Sequences of the PCR-amplified DNA fragments were then used to perform BLAST searches on the GenBank nucleic acid database (National Center for Biotechnology Information website, <http://www.ncbi.nlm.nih.gov/BLAST/>) for species identification.

Phylogenetic analysis

Multiple alignment of the ITS2 sequences with those of related species available in the GenBank library was performed using the CLUSTALW program [30]. Phylogenetic trees were produced using the neighbor-joining (NJ) program with Kimura's two-parameter model [31] on MEGA X version 10.2.2 [32]. The statistical significance of the resulting NJ trees was evaluated using a bootstrap test with 1,000 replications.

Thirty-eight specimens (37 from Japan and one from Vietnam), made up of five species, out of the total collected mosquitoes were used for phylogenetic analyses. The corresponding sequence data for eight strains of six species: *An. anthropophagus* Xu and Feng strain SMMU-FK1 from China (GenBank accession no. AY803792) [33], *An. belenrae* strain isolate 3 from South Korea (AY375466) [34], *An. kleini* Rueda from South Korea (DQ177501, direct submission to the GenBank database), *An. lesteri* strain specimen 1 (type B) and specimen 2 (type C) from South Korea (AJ620899 and AJ620900, respectively) [25], *An. sinensis* strain isolate 1 from South Korea (AY375464) [34], and *An. sineroides* strain SINEK02 from South Korea (GU384724) [35], served as reference strains. *Anopheles yatsushiroensis* from China (AY186792) [22], which was renamed *An. pullus* M. Yamada [36, 37], was included as an outgroup sequence.

Distribution map

The distribution map was constructed using ArcGIS 10 (ESRI Inc., Redlands, CA), and information from the Geographic Information System.

Results

Phylogenetic analysis

A total of 248 specimens (181 adults and 67 larvae) were collected in Hokkaido between 2001 and 2015. The collected specimens were classified into four anopheline species of the *An. hyrcanus* group; *An. belenrae*, *An. engarensis*, *An. lesteri*, and *An. sineroides* (Table 1). Interestingly, *An. sinensis* was not collected from Hokkaido during our survey. Phylogenetic analysis was performed using the 485bp ITS2 sequence of 38 specimens, collected from different sites (30 from Hokkaido, seven from Japanese regions outside Hokkaido, and one from Vietnam) in different years, and eight reference sequences from the GenBank database (Table 2).

The NJ phylogenetic trees revealed five robust clades, consisting of the four species listed above and *An. sinensis* (Figs. 1 and 2). Unfortunately, *An. sinensis* was not detected in Hokkaido in this study. Therefore, we analyzed *An. sinensis* collected from areas outside Hokkaido. The cluster of *An. sinensis* showed small differences (Figs. 1 and 2). Nonetheless, there were no differences between the four Japanese strains of *An. sinensis* (Yokohama08, Echizen379, Kaifu353, and Misawa391) and the strain from South Korea (isolate 1).

In 2004, two larvae morphologically identified as *An. sinensis* were confirmed to be *An. belenrae* using the ITS2 sequence, marking the first record of *An. belenrae* in Japan (Akan44 strain). Subsequent phylogenetic analysis showed that *An. belenrae* was the closest related species to *An. sinensis*, followed by *An. engarensis* (Figs. 1 and 2).

Intra- and interspecific ITS2 variation

The levels of nucleotide variation detected between pairs of specimen in the *An. hyrcanus* group are presented in Table 3. There were no genetic differences between the 10 Japanese strains of *An. belenrae* (Akan44, Kushiro10, Kushiro201, Kushiro313, Kushiro418, Kawakami60, Akan712, Kushiro503, Nakagawa807, and Nakagawa26) and the Korean strain (isolate 3), with a 0% pairwise divergence (Figs. 1, 2 and Table 3). This suggests that the Japanese *An. belenrae* and the Korean *An. belenrae* are the same at least based on the ITS2 sequences. The Vietnamese strain (GLVN59) showed slight differences from the other strains, with a 0.26% pairwise divergence (Table 3). Regarding the clusters of *An. engarensis*, one specimen (Daisen55) collected in Akita Prefecture, an area outside Hokkaido, was slightly different from the nine specimens collected in Hokkaido (Abashiri02, Akashuri18, Yubari25, Yufutsu20, Kameda40, Yufutsu115, L1532, A14-22, and L1468) (Figs. 1 and 2). No genetic differences were observed among the *An. engarensis* specimens, except for the Daisen55 strain, with a 4.63% pairwise divergence from the others (Table 3).

As mentioned above, the intraspecific variation between these three species was very low (0%, 0.26%, and 4.63% in *An. belenrae*, *An. sinensis*, and *An. engarensis*, respectively) (Table 3); a few differences were found based on the collection areas. Regarding the interspecific variation, pairwise divergence between *An. belenrae* and two other anopheline species (*An. sinensis* and *An. engarensis*) was 13.25–13.57% and 13.27–14.26%, respectively, and 13.86–15.19% between *An. sinensis* and *An. engarensis* (Table 3). The values among these three species indicate high levels of genetic differentiation. The NJ phylogenetic trees also showed that one strain of *An. kleini* from South Korea was located closer to *An. engarensis* than to *An. belenrae* and *An. sinensis* (Figs. 1 and 2). A detailed study of the genetic background of these species will be necessary.

In *An. sineroides*, no differences were found between the two specimens from Hokkaido (Fukagawa13 and Kushiro343), and the one from South Korea (SINEK02) (Figs. 1, 2, and Table 3). However, the two specimens from areas outside Hokkaido (Hida386 and Toyama80) showed few differences from the three mentioned above, with 0.26 and 0.8% pairwise divergences (Table 3). In contrast, a large intraspecific variation was observed in *An. lesteri* (Figs. 1 and 2). Pairwise divergence was in the range of 0.26–2.14% among the nine strains from Hokkaido (Abashiri01, Abashiri17, Abashiri42, Hakodate31, Kamiiso35, Kameda38, Kushiro317, Kushiro505, and A2651) (Table 3). The two Korean strains (specimen 1 and specimen 2 classified as Type B and Type C of *An. lesteri*, respectively) were quite far from the other *An. lesteri* strains (Figs. 1 and 2). Pairwise divergence among the 11 *An. lesteri* strains ranged from 0.26–8.96%, indicating that *An. lesteri* appeared to form a highly divergent population. The cluster of *An. lesteri* revealed a low pairwise divergence, ranging from 0–2.14%, between the nine *An. lesteri* strains

from Japan and the Chinese strain of *An. anthropophagus* (SMMU-FK1) (Table 3), suggesting that they may belong to the same species.

Discussion

The first record of *An. belenrae* in Japan

Our surveys from 2001 to 2015 revealed a significant change in the distribution range of the *An. hyrcanus* group in Hokkaido reported in the 1980s [3–6], including the first record of *An. belenrae* in Japan. Two larvae collected in the Kushiro Wetland in 2004 were tentatively named *An. sinensis* Kushiro strain, based solely on the morphological characteristics of the emerged adults. However, phylogenetic trees constructed using ITS2 sequence revealed that this *An. sinensis* Kushiro strain formed a robust clade that was clearly different from the clades of *An. sinensis* and other *Anopheles* species. Interestingly, the ITS2 sequence of the Kushiro strain was not identical to that of the *An. sinensis* strains collected in southern Japan, outside Hokkaido but to that of *An. belenrae*, a new strain reported in South Korea in 2005 [24]. The Kushiro strain could confidently be included in the *An. belenrae* cluster because of the absence of intraspecific divergence as mentioned above. This species was consistently found in the Kushiro Wetland after the first detection in 2004. In contrast, *An. belenrae* was not found outside Hokkaido in our 15-year nationwide survey. Thus, we concluded that this species is restricted to the Kushiro Wetland in Hokkaido.

The Kushiro Wetland is the largest marshland/wetland in Japan and is located in the Kushiro Plain. The Kushiro Wetland has been the focus of nature conservation efforts since before World War II, was registered as a Ramsar site in 1980 and designated as a national park in 1987. It is also famous for being the breeding ground for Japanese Cranes, *Grus japonensis*, and many other wild birds and a protected area for natural monuments, birds, and animals; thus, land development is strictly regulated. In South Korea, *An. belenrae* is found in the northern part of the country near the border with North Korea [24, 38, 39]. In China, *An. belenrae* is reportedly distributed in Shandong and Liaoning Provinces in northeast China, facing the Korean Peninsula [40]. These areas are not only geographically close to Japan, but may also have similarities in climate, vegetation, and some environmental factors with the Kushiro Wetland. However, further investigation is needed to compare the morphological characteristics of Japanese and Korean *An. belenrae*, and to determine the distribution of this species in locations outside Hokkaido in Japan. We hope that ecological and evolutionary factors impacting the emergence of *An. belenrae* will be elucidated with the development of molecular biological technology.

No information of *An. sinensis* from Hokkaido

The next noteworthy finding was the disappearance of *An. sinensis* from Hokkaido. In previous surveys, *An. sinensis* was generally distributed throughout Hokkaido [3–6] (Fig. 3). Although it is often found in the same larval habitat as *An. lesteri*, it is thought to occur more frequently in developed paddy fields and swamps [41]. In the 2000s, we did not find any *An. sinensis* in the habitat of *An. lesteri*, nor did we find any new sources or habitats (Fig. 4). It is possible that the larval habitat of *An. sinensis* changed drastically in the 20-year period between the previous studies [3–6] and this current study. For example, in

the 1949 [1] and 1976 [6] surveys, four members of the *An. hyrcanus* group were detected in northeastern Hokkaido, around Rubeshibe (Fig. 3). At that time, there were paddy fields all over the district, and forestry and horse-logging were the main industries. In recent times however, the horse-logging industry has declined drastically, and the paddy fields have been replaced with upland crops. Furthermore, neither *An. sinensis* nor *An. sineroides* was found in this area, around Ozora, during our survey (Fig. 4). It is highly likely that the changes in vegetation and industry have affected the distribution of anopheline mosquitoes.

There may be other reasons for the disappearance of *An. sinensis* from Hokkaido. The classification of organisms was mainly based on morphological keys until the 1990s. Although adults of *An. belenrae* can be separated morphologically from those of *An. lesteri*, *An. sinensis* and other species [24], it was likely that *An. belenrae* and *An. sinensis* could not be differentiated morphologically. Therefore, it should be noted that *An. belenrae* may have been classified as *An. sinensis*. The results from the ITS2 sequences in this study revealed that these two species were genetically the closest related. In addition, the pairwise interspecific distance in mitochondrial genomes calculated by each fragment showed minor or no difference between *An. sinensis*, *An. belenrae* and *An. kleini* [40]. Phylogenetic analysis of COI indicated that ancient hybridizations probably occurred among these three closely related species [42], making differentiation with the COI sequence improbable. To address this problem, we tried to extract DNA and decipher the nucleotide sequence from age-old, dried specimens previously classified as *An. sinensis* collected in Hokkaido [3–6]. However, no new information could be obtained from these specimens. We hope that techniques for genetic analysis using age-old specimens will be developed as soon as possible.

Anopheles engarensis in Japan

Anopheles engarensis is also a species whose distributional range has reduced in Hokkaido. This species, first described in Engaro-cho (North-eastern Hokkaido) in 1977, [3] was also found in Monbetsu, Kushiro, and Obihiro until 1984 [6], suggesting a wide distribution in Hokkaido [3–6] (Fig. 3). However, our surveillance found this species to be restricted to western and southern Hokkaido (Fig. 4). In addition, this species was also collected in northern Tohoku, Akita Prefecture, suggesting a southward shift presumably due to changes in the environment, including the climate of larval habitats. In terms of classification, *An. engarensis* was recognized as a new species in the *An. hyrcanus* group only after its chromosomal structure was determined to be different from *An. sinensis* [4]. This was because of the high morphological similarity between the two species. Indeed, the only distinguishing feature was the unique number of clasper movements of *An. engarensis* males during artificial mating, a common method for laboratory maintenance of anopheline mosquitoes [5].

In general, ITS2 is known to have high interspecific and low intraspecific variability; however, extensive intraspecific variations have been reported in anopheline mosquitoes. For instance, ITS2 intraspecific variations ranged from 0.2–19.0% for the Latin American anophelines [43]. In the *An. hyrcanus* group, the average intraspecific distance was 0.3%, but no intraspecific variations were observed in *An. belenrae* [42]. These results suggest that the ITS2 spacer is a good marker for differentiating between members of

the *An. hyrcanus* group. In this study, there were no intraspecific variations in the *An. belenrae*, *An. engarensis*, and *An. sineroides* strains from Hokkaido. However, there was significant intraspecific variation between the nine *An. engarensis* strains from Hokkaido and the Daisen55 strain from Akita Prefecture. The genetic distance of 4.7% was considerably greater than the 0.22% intraspecific variation in the *An. sinensis* strains from Vietnam, South Korea, and Japan. We inferred that the Daisen55 *An. engarensis* strain was not introduced from Hokkaido but inhabited the Tohoku region independently. On the other hand, in species groups consisting of recently diverged members, such as the *An. gambiae* complex, the interspecific differences in ITS2 were reported to be minor, ranging from 0.4–1.6% [20]. It is possible that *An. engarensis* is a recently diverged lineage.

Anopheles lesteri in Hokkaido

In Rubeshibe Hokkaido, at least seven cases of falciparum malaria were recorded between 1946 and 1947. An unsuccessful survey was conducted to determine the vector mosquitoes involved in the transmission although *An. sinensis* and *An. sineroides* were collected [1]. During the falciparum malaria epidemic in the vicinity of Guangdong City, China, around 1942, the transmission was inferred to have involved *An. lesteri* and not *An. sinensis*. This inference was based on results from field investigations and subsequent infection experiments with *Plasmodium falciparum* [44]. Based on this inference, it was suggested but never confirmed that *An. lesteri* may have been involved in the outbreak of falciparum malaria in Rubeshibe, Hokkaido. In terms of distribution, *An. lesteri* which was initially thought to be restricted to western Islands of Japan, such as the Kyushu Island [44], was also found in various areas of Honshu mainland of Japan [12], Hokkaido [45], Okinawa Island, and Yaeyama Islands [46]. The present survey confirmed that *An. lesteri* is still widely distributed in Hokkaido (Figs. 3 and 4). At the start of our survey in 2001, we noticed female mosquitoes collected in Ozora, Hokkaido, to have an intense affinity for human blood. These female mosquitoes were therefore considered, and subsequently confirmed, to be *An. lesteri* based on the reported high anthropophilic nature of *An. lesteri* relative to *An. sinensis* and other members of the *An. hyrcanus* group [12, 47]. We therefore expected to easily collect *An. lesteri* in subsequent surveys in Hokkaido.

In our study, the ITS2 intraspecific distance in *An. lesteri* ranged from 0–9.44%. These values suggest that *An. lesteri* is a highly divergent species when *An. lesteri* type B (specimen 1) and type C (specimen 2) from South Korea are included in this species. Since the ITS2 distance of this species varies even within Hokkaido, there is a possibility that *An. lesteri* includes crypto-species. In defining this species, it is necessary to analyze both the COI barcoding region and the ITS2 region. Moreover, a large number of specimens, collected outside Hokkaido, will be necessary. In a previous study, a short interspecific distance of 7.2% was observed between *An. kleini* and *An. engarensis* [42]. We obtained similar ITS2 distances of 6.47% and 8.67% between *An. kleini* and our *An. engarensis* specimens from Hokkaido and *An. kleini* and *An. engarensis* Daisen55 strain, respectively. Although these results may provide validation that *An. kleini* is a synonym of *An. engarensis*, further analysis is required. We also presented evidence that *An. anthropophagus* and *An. lesteri* were conspecific, based on the ITS2 divergence between them. Our results based on interspecific comparisons of ITS2 divergence may also support previous reports that

An. belenrae and *An. sinensis* are genetically distinct [24, 25], and *An. anthropophagus* is a conspecific species of *An. lesteri* [34, 48].

Conclusions

ITS2 sequence divergence clearly disclosed the current distribution of the *An. hyrcanus* group mosquitoes in Hokkaido, demonstrating great differences from surveys conducted between 1969 and 1984. In particular, the area inhabited by *An. sinensis* has greatly reduced, and the newly discovered *An. belenrae* was confirmed to inhabit only eastern Hokkaido. In summary, this study showed that Hokkaido harbored four members of the *An. hyrcanus* group, namely *An. engarensis*, *An. belenrae*, *An. sineroides*, and *An. lesteri*.

Here, we have demonstrated the presence of two anopheline species that have been reported as malaria vectors, *An. belenrae* instead of *An. sinensis*, in addition to *An. lesteri*, in Hokkaido since 2001. Although the malaria vector capacity of the Japanese strain of *An. belenrae* has not yet been evaluated, the Korean strain is considered to be a vector or potential vector of *P. vivax* [24, 49]. Fortunately, all recently reported cases of malaria in Japan have been imported. However, emergence of potential autochthonous malaria epidemics should always be of concern because multiple malaria vector species still remain in Japan, as confirmed in this study.

Abbreviations

NIID: National Institute of Infectious Diseases; ITS2: Internal Transcribed spacer 2; COI: cytochrome oxidase subunit 1

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The newly generated sequences were submitted in the GenBank database under the accession numbers LC634739–LC634772.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

KS, TK, KT, NN and MK conceived and designed this study. KS, NI and YM drafted the manuscript. KS and NI conducted phylogenetic analyses. YM and NN analyzed GIS information and drew the distribution maps. YH, KSK, KT, YT and TH contributed to facilitation in field investigations. All authors participated in field investigations. All authors contributed to revise the manuscript and approved the final manuscript.

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Tables

Due to technical limitations, table 1 to 3 is only available as a download in the Supplemental Files section.

Figures



Figure 1

Phylogenetic relationships among members of the *Anopheles hyrcanus* group. Neighbor-joining (NJ) phylogenetic tree was constructed based on partial ITS2 region nucleotide sequences. A distance matrix was calculated using the Kimura's two-parameter evolutionary model and the tree was constructed using the NJ approach in MEGA X ver. 10.2.2. The scale bar indicates the proportion of sites changing along

each branch. The numbers on the internodes indicate percentages of 1,000 bootstrap replicates. Bootstrap values <70 are not shown in this figure. This phylogenetic tree is a rooted and traditional rectangular tree with *An. pullus* (AY186792) as an outgroup sequence. All specimens collected in the present study are marked with circles. The closed circles mark mosquito specimens collected from Hokkaido, Japan, and open circles mark specimens from the areas outside Hokkaido. Abbreviations of strains and sequence accession numbers of specimens used in this study are listed in Table 1.

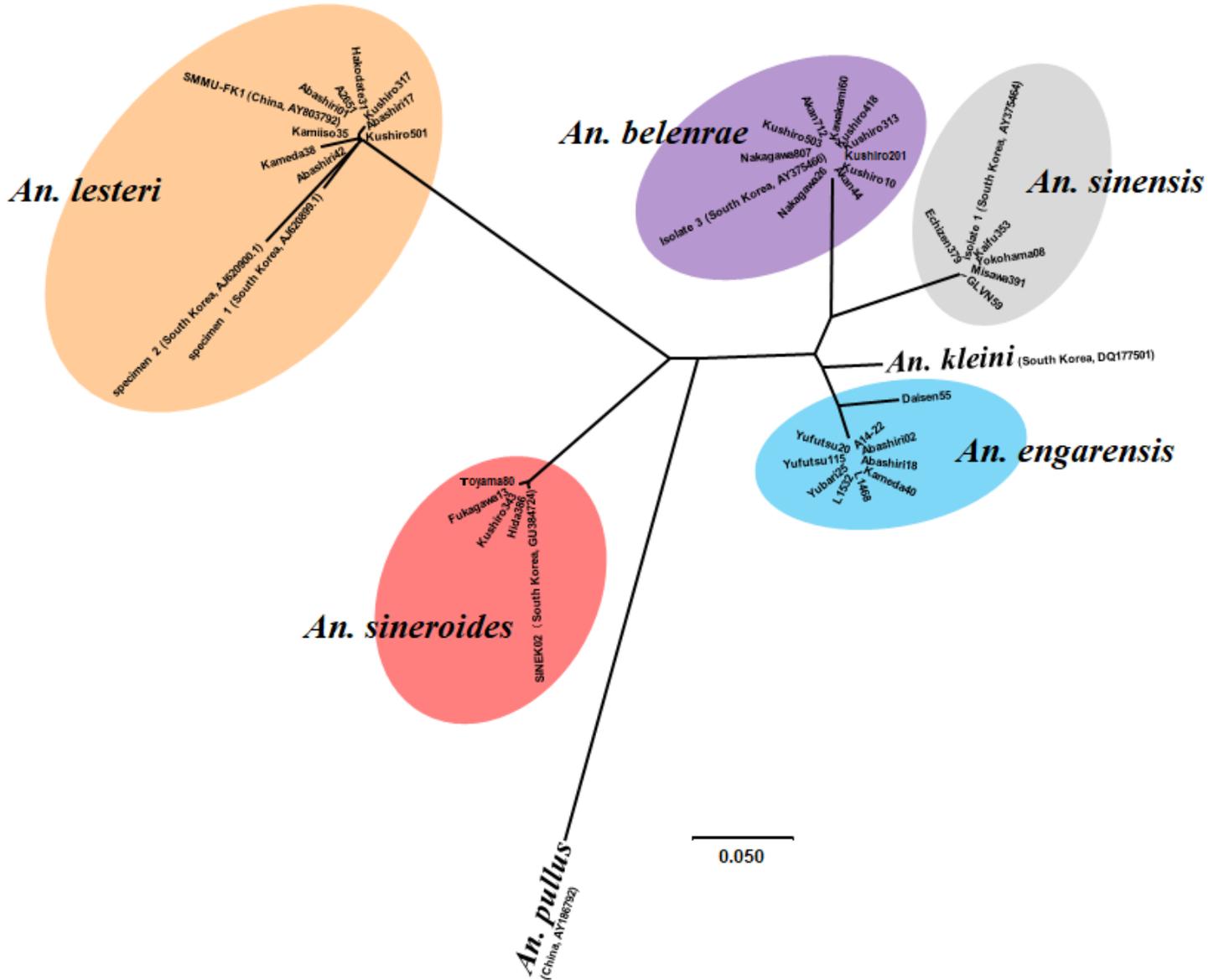


Figure 2

Phylogenetic relationships among members of the *Anopheles hyrcanus* group. Neighbor-joining (NJ) phylogenetic tree were constructed based on partial ITS2 region nucleotide sequences. A distance matrix was calculated using the Kimura's two-parameter evolutionary model and the tree was constructed using the NJ approach in MEGA X ver. 10.2.2. This phylogenetic tree is a rootless and radiation tree with *An. pullus* (AY186792) as an outgroup sequence to know more closely related species. The scale bar indicates the proportion of sites changing along each branch. Bootstrap values are not indicated in this

figure. The species names in this group are bold. The members in the same species are shaded. Abbreviations of strains and sequence accession numbers of specimens used in this study are listed in Table 1.

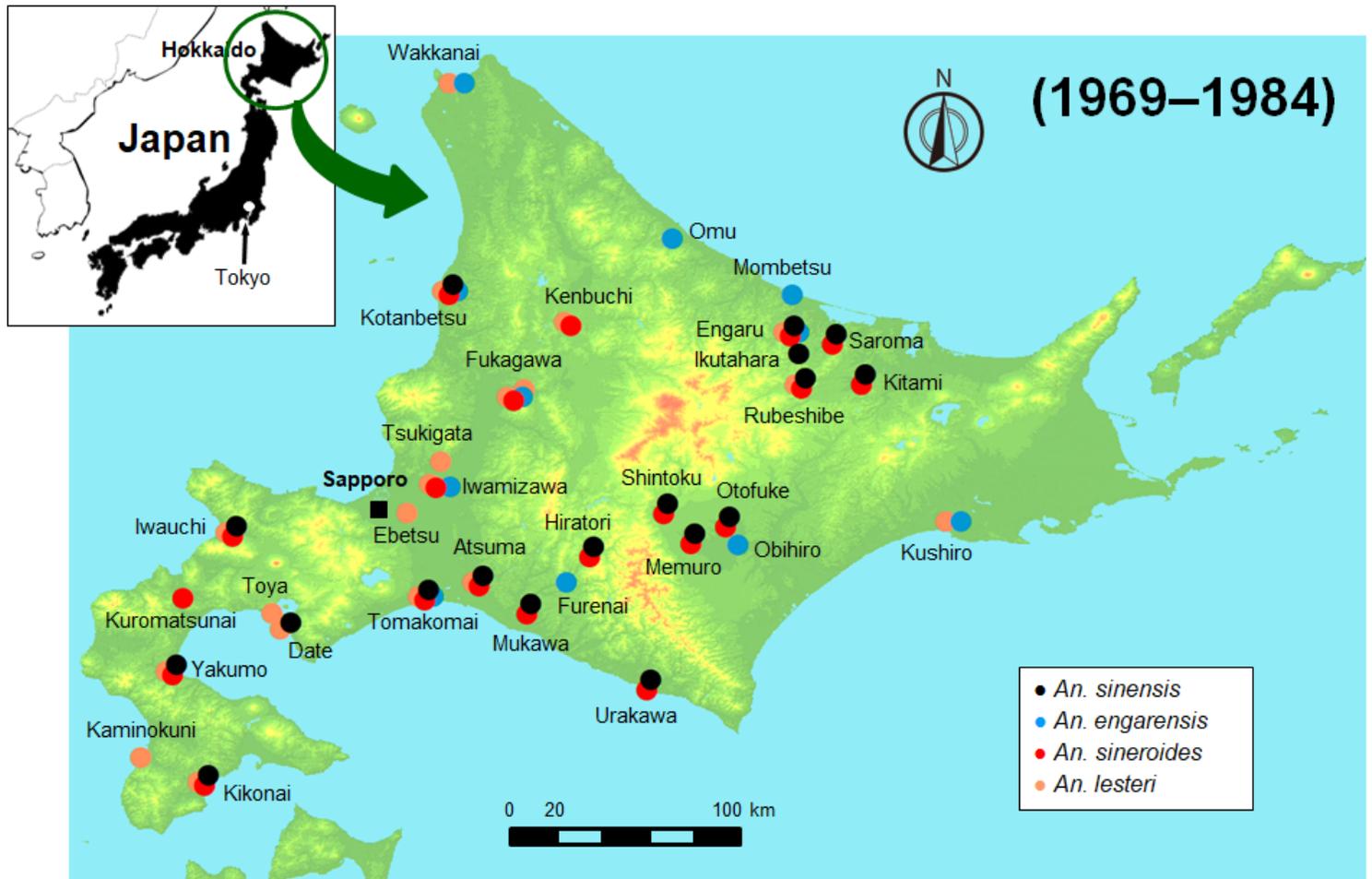


Figure 3

Distribution map of the *Anopheles hyrcanus* group mosquitoes in Hokkaido, Japan, as confirmed by surveys conducted during 1969–1984. This map was created from references [3–6]. This map shows mosquito species collected from 29 sites. Black, blue, red, and orange circles indicate collection sites of *An. sinensis*, *An. engarensis*, *An. sineroides*, and *An. lesteri*, respectively.

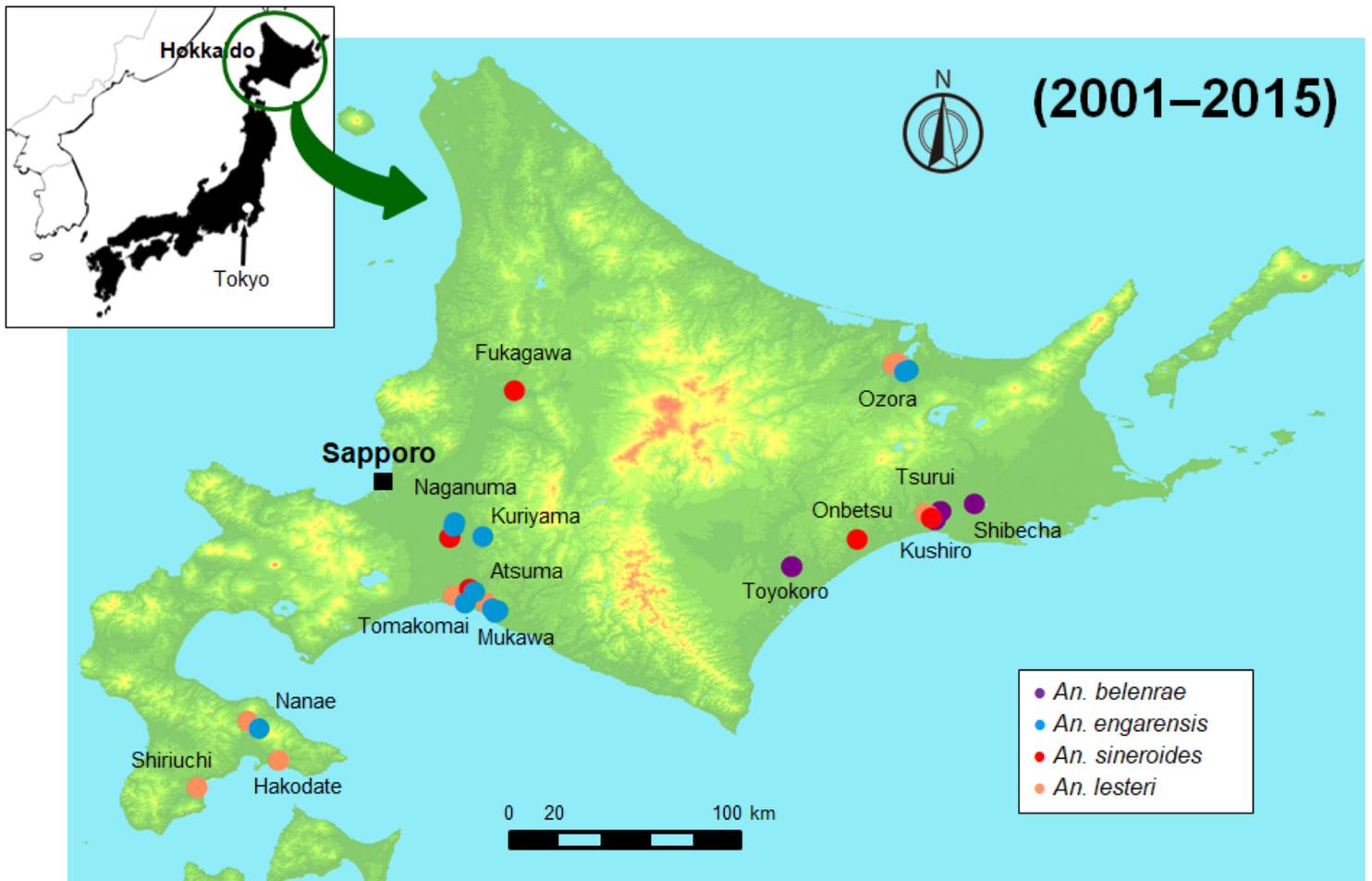


Figure 4

Distribution map of the *Anopheles hyrcanus* group mosquitoes in Hokkaido, Japan, as disclosed by the present study conducted during 2001–2015. This map shows mosquito species collected from 15 collection sites. Purple, blue, red, and orange circles show collection sites of *An. belenrae*, *An. engarensis*, *An. sineroides*, and *An. lesteri*, respectively.

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